STUDIES IN EPIDEMIC POLIOMYELITIS

I. THE ISOLATION AND CULTIVATION OF THE GLOBOID BODIES

PLATE 5

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In 1913, Flexner and Noguchi isolated a minute, anaerobic micro-organism from the nervous tissues of human beings and monkeys dying of acute poliomyelitis. They were uncertain regarding the nature of these organisms and referred to them as "globoid bodies." They cultivated the micro-organism with success, inoculated pure cultures into the brains of monkeys and produced clinical and anatomic poliomyelitis; from the nervous tissues and blood of the infected monkeys the globoid bodies were recovered in pure culture.

In this paper we wish to record the isolation from human and monkey poliomyelitic material of 4 different strains of an organism which morphologically and culturally agrees in every particular with the descriptions of the globoid bodies of Flexner and his associates.

In January, 1917, we began the work on 5 lots of glycerolated human poliomyelitic nerve tissue, obtained during the epidemic in the summer of 1916, at the Philadelphia Hospital for Contagious Diseases. The tissues had been in 50% glycerol for 5 months. With the writings of Flexner and his associates before us, we proceeded to follow the methods described by them with the utmost exactness. The tissue was emulsified, filtered through paper and some specimens were passed through Chamberland and Reichel filters. The filtrates were distributed among tubes of ascitic fluid to which fragments of sterile rabbit kidney had been added. Two c.c. of filtrate was placed in each tube. The fluid was overlaid with a layer of sterile paraffin oil approximately 2 inches deep and the tubes placed in the incubator at 37 C. As means for producing an atmosphere of hydrogen were not available at this time, these tubes were not placed in an anaerobic jar. Within a few days, a few tubes became clouded and were found to contain gross contaminations and were discarded. The rest of the tubes remained perfectly clear. At the end of a month 1 tube inoculated with filtrate of a brain and in 1 inoculated with filtrate of a spinal cord a faint haze was detected about the kidney fragment. The haze slowly

Received for publication October 23, 1917.

* In the Laboratory of Experimental Pathology of the University of Pennsylvania the work was conducted with the cooperation of Drs. Allen J. Smith, Charles K. Mills and others. as part of a series of investigations bearing on the microparasitology and clinical aspects of poliomyelitis.

grew up the tube to a point 2 cm. above the kidney and stopped abruptly, the fluid above remaining quite clear. A drop of the clouded fluid was withdrawn with a capillary pipet, and stained with Giemsa as described by Flexner and Noguchi. The films showed a few small, violet colored masses which, while they suggested groups of minute cocci, lacked any very distinct morphologic characteristics. No similar masses could be found in control tubes. Other tubes of kidney-ascitic fluid were inoculated from the original two. In 5 days these second tubes showed a faint haziness around the kidney and again smears showed the violet clusters which could not be positively identified as organisms. In addition to these violet masses a few large round, single and paired cocci were found which stained a deep blue. Nothing in the nature of transition forms between the two types of bodies could be observed. The clusters appeared to be made up of very small bodies, all of the same size and of a uniform violet color, whereas the cocci were large and distinctly blue. A third generation of tubes, planted from the second, clouded up uniformly to the oil after 24 hours in the incubator and were found to be filled with large, short chain streptococci. The violet clusters did not reappear. The large streptococci when transferred to broth and agar slants grew readily aerobically and, by methods detailed elsewhere by two of us, were proved to be identical with streptococci very commonly found in many localities associated with poliomyelitis. By no methods of aerobic or anaerobic cultivation we could devise were we able to force this large, short-chain streptococcus to resemble in any particular the cluster of very minute violet bodies we had seen in the first and second generation. The streptococci remained streptococci. We filtered cultures of these streptococci through the candles used originally for the brain and cord emulsion. A few of the streptococci evidently passed through; for some subcultures of the filtrates clouded up and some did not. It seemed to us probable that two distinct organisms had been present in the poliomyelitic tissue and had passed through the Chamberland and Reichel filters. The ones which were seen in violet masses in smears from the original tubes had grown well under the anaerobic conditions, while the large blue cocci apparently had been unable to multiply until oxygen had been carried down into the fluid by the pipets used for drawing off portions for examination and transplanting, and by shaking, unavoidable in such manipulations. With this possibility in mind we returned to the original tubes. Here, too, the streptococci had now grown abundantly and clouded the ascitic fluid up to the oil. We diluted these original tubes slightly with normal salt solution and passed them through a Mandler filter. The Mandler, a diatomaceous earth filter of American manufacture, is graded by immersing the candle in water for 24 hours and then determining the pressure necessary to force air through the pores while it is still under water. The one we used withstood 11 lbs. of pressure. Tubes of kidney-ascitic fluid were inoculated with 2 c.c. of these second filtrates, covered with a layer of oil and placed in a tall museum jar in the lid of which holes had been drilled and rubber tubes attached. The lid was sealed with vaselin and clamped down and hydrogen from a tank run in until pure hydrogen escaped from the outlet. While this undoubtedly did not remove all the oxygen it removed a large part of it. The tubes remained clear for two weeks. At the end of that time when they were carefully examined against a black background it was possible to distinguish a very faint haziness which stopped abruptly 1 or 2 cm. below the oil. The column of haze was thus considerably higher than when the hydrogen atmosphere had not been used. Smears stained as before with Giemsa showed very little. On an entire slide but 1 or 2

violet clusters, lacking definite morphology, could be found. The large blue cocci were not present nor did they again appear in any later subculture. When the fluid was examined under a dark field apparatus nothing resembling an organism could be seen among the hosts of dancing granules. It was not until we resorted to the examination of a simple hanging drop, using the oil immersion objective, that we saw anything that could be said to have a distinct organized morphology. By making a hanging drop with fluid drawn from around the rabbit kidney, reducing the illumination by lowering the Abbé condenser, and carefully focusing up and down in the drop certain tiny globular bodies could be seen in the fluid drawn from the inoculated tubes which were not found in fluid drawn from the control tubes. These bodies were exceedingly minute. Pneumococci added to the drop for comparison seemed gigantic beside them. After 4 or 5 days' incubation, pairs and threes of the bodies could be found—never many; several fields might be examined and only one or two groups seen. In fluid from both inoculated and uninoculated tubes tiny single bodies showing active brownian movements could be seen, but the grouping in pairs, chains and, later, clusters was characteristic of the inoculated tubes. In fluid from tubes 7-10 days old short chains of 10 or 12 globules were seen. These chains were often tightly coiled up and the organisms were of such size that the entire chain might have been enclosed in the capsule of a pair of pneumococci. Still later tiny clusters with outgrowing chains could be found. At that time several such clusters might be found in a field. Examining our cultures in this manner in the hanging drop we were able to watch the slow development of the organism. When the organisms were fairly abundant, a new tube was inoculated with about 1 c.c. and the process followed in the next generation. One of our strains, No. 2b, is now in the tenth generation. Six months were required to accomplish this number of transfers. In the later generations the organisms multiplied more rapidly and with more certainty. Of all the tubes used in the 10 transfers it was only the occasional one that showed a perceptible haze on inspection. The haze was recognizable owing to the fact that it ended some distance below the oil with a sharp line of demarcation: as soon as this line was broken by the pipet or by slight shaking in handling, the haze would become distributed throughout the tube and no longer recognizable. Tubes of medium prepared in exactly the same manner would differ slightly in their optical density. The haze due to the globular organisms was so slight that a control tube might look more clouded than the inoculated tube, but when comparison could be made between the upper and lower portions of the same column the difference could be recognized.

When the ninth subculture had grown well, tubes of semi-solid medium were prepared by first inoculating a tube of kidney-ascitic fluid (8 c.c. of fluid) as usual and then adding 5 c.c. of 2% agar, melted and cooled to 40 C. In this medium a faint haziness appeared which ended abruptly a short distance below the oil. This cloud remained visible, as the solidity of the medium prevented its being distributed throughout the tube.

During the first six months in only one or two instances did we succeed in staining the organisms satisfactorily. Smears made from tubes in which numbers of the globoid bodies could be seen in the hanging drop, and stained according to the method described by Flexner and Noguchi, either showed no organisms whatever or a very few faintly stained dots hardly to be distinguished in the deeply stained background. Before we adopted the hanging-drop method of examination of our cultures many tubes which remained clear and in which no staining organisms were found were pronounced sterile. It now seems probable that many of these tubes contained the globoid bodies but that they

were overlooked. Later we found a method whereby the globoid bodies could be stained with ease. A subculture from the third generation was made in a 100 c.c. Ehrlenmeyer flask of combined solid and fluid medium by the method described by Noguchi. This was placed in the anaerobic jar and the organisms allowed to grow for two months. At the end of that time the ascitic fluid and agar remained clear, but a slight whitish sediment covered the surface of the agar. In the hanging drop the fluid was found to be crowded with clusters and large masses of the globoid bodies. The fluid was drawn off and centrifugated at high speed for two hours and the sediment washed with physiologic sodium chlorid solution and again given a prolonged centrifugalization. The final sediment was taken up in salt solution and yielded 3 c.c. of a heavy white suspension.

When smears were made from these washed organisms it was found that they stained readily with many stains. The films were fixed in methyl alcohol for 1 hour or in formalin vapor for 5 minutes. Giemsa stain, diluted 1:10 with distilled water and applied for 1 or 2 hours, yielded the most satisfactory results. Carbolthionin and carbolfuchsin, diluted and used in the same manner, stained the washed organisms with great distinctness. Loeffler's methylene blue undiluted was taken up less readily. In our experience the washed organisms have been decolorized by Gram's method. The washed globoid bodies were readily phagocytized after having been digested with normal human serum.

Later we succeeded in staining the organism in the ascitic fluid by making a thin film and drying over-night in the incubator. The film was fixed in methyl alcohol for 1 hour and transferred to Giemsa, diluted 1:10 with distilled water, and the jar placed in the paraffin oven at 50 C. for 24 hours. But even by this method the globoid bodies were stained distinctly in only a small proportion of films and in many films were not stained at all. We have used two lots of Giemsa stain, both prepared from Grubler's powder, one more than a year old and one quite fresh. With the older stain the globoid bodies stained violet, while with the fresh stain the color was usually more bluish. Whether this difference was due to the age of the stain or to something in the preparation we are unable to say. Flexner and Noguchi state that the globoid bodies at times stain violet with Giemsa and at times bluish.

A stained film shows the minute organisms without particular arrangement. In the film made from a young growth the bodies are of the same size, and take the stain uniformly. In old cultures degeneration-forms appear. Some individuals in a group do not stain and some are slightly larger than the others. The largest barely approach the size of a small coccus.

In addition to the two original strains from human material we have isolated two other strains from monkeys with experimental poliomyelitis. Cultures were made of the brain and cord of a monkey which developed paralysis of one arm and showed typical poliomyelitic changes following the intracerebral injection of human virus sent to the University of Pennsylvania by Dr. Leake from a patient in West Virginia succumbing during the winter epidemic of 1917. Twenty tubes of kidney-ascitic fluid were inoculated with small pieces of brain and cord. A layer of oil was placed over the fluid but the tubes were not kept in an atmosphere of hydrogen. After 6 days' incubation 9 of the tubes were faintly and uniformly clouded up to the layer of oil. Subcultures into broth gave pure cultures of the same large, short-chain streptococcus we had found in the human poliomyelitic material. The balance of the tubes remained clear and repeated subcultures from them failed to show any growth. Thirty-three days after the cultures were made, a very faint haze was observed about the kidney

in two of the tubes which had remained clear up to that time and which had given repeated negative cultures in broth. The cloud did not ascend higher than 1 cm. above the kidney. Aerobic cultures from this cloud in blood broth and blood agar showed no growth. In the hanging drop, however, the globoid bodies were recognized in fluid from these 2 tubes. Numerous successful transfers of this strain into tubes of kidney-ascitic fluid have been effected. One tube was accidentally contaminated with a small gram-negative bacillus, probably B. proteus. The contaminating bacillus was removed by filtration through a Mandler filter and the globoid bodies grown from the filtrate.

Our second monkey strain was isolated from a piece of the spinal cord of a monkey showing typical poliomyelitis. We were furnished with the fragment of cord by the Pennsylvania State Department of Health Laboratory, through the courtesy of Drs. Samuel G. Dixon and James B. Rucker, Jr. It had been in glycerol for 5 months when used. The fragment, about 1 cm. long was emulsified and the emulsion passed through a Mandler filter and the filtrate planted in four tubes of kidney-ascitic fluid. These were overlaid with paraffin oil and placed in the hydrogen jar. In 6 weeks a faint haze was observed extending part way up one tube and in addition a fine granular precipitate was present. Hanging drop examiation showed a few globoid chains and clusters and a slender bacillus. The fluid in this tube was refiltered and the bacillus did not reappear in cultures from the filtrate, while the globoid bodies grew well.

In preparing 1 lot of kidney-ascitic fluid medium the fragments of rabbit kidney were placed alone in the test tubes and the basket containing them put on ice. Some weeks elapsed before the ascitic fluid was added, at which time the kidney fragments had become darkened. During the incubation to test the sterility of the lot, a small amount of hemoglobin diffused upward through the fluid. When these tubes were used for subcultures we were surprised to find that the globoid bodies grew more readily than in tubes in which fresh kidney fragments had been used. It seems possible that allowing the kidney fragments to age before adding the ascitic fluid may create a more favorable medium for the globoid bodies. Both of our successful isolations from monkey poliomyelitic material were obtained in tubes in which the kidney had been aged in this manner.

Successful isolations have been performed with two samples of ascitic fluid, and a third has proved successful in supporting growth of subcultures. It may be possible, therefore, that the variation among samples of ascitic fluid has less influence on the growth of the organism than has been supposed.

SUMMARY

A micro-organism has been isolated from human and monkey poliomyelitic nervous tissue which agrees morphologically and culturally in every particular with the globoid bodies first described by Flexner and Noguchi. Four different cultures have been obtained: two from human and two from monkey poliomyelitic tissue.

It has been observed that the macroscopic appearance of tubes of kidney-ascitic fluid medium which contain good growths of this organism differs from that of the uninoculated control tubes to such a slight degree that often inspection of the tube alone is of no value in determining the presence or absence of a growth.

The organism while in the ascitic fluid stains with such difficulty and in the earlier generations the minute globules are so few in number, that they may easily escape observation if stained smears are relied on to detect growth.

Careful examination in the hanging drop has proved a simple and rapid method of detecting the organism in fluid medium.

The organisms which stain with difficulty when in the ascitic fluid are stained with ease after they have been freed from the culture medium by washing in normal salt solution.

A more rigid anaerobiosis than can be obtained by the use of a layer of paraffin oil is advisable for the successful cultivation of this microorganism.

The use of rabbit kidney which has been allowed to age before adding the ascitic fluid may be one factor in creating a successful artificial medium for the cultivation of the organism.

EXPLANATION OF PLATE 5

FIG. 1. Washed globoid bodies. Streptococcus pyogenes has been stained on the same slide to show comparative size. Giemsa stain. $\times$ 1200.

FIG. 2. Washed globoid bodies. Pneumococcus has been stained on the same slide to show comparative size. Giemsa stain. $\times$ 1200.

FIGS. 3 AND 4. Washed globoid bodies and Rosenow's streptococcus. Pure cultures of each organism were smeared and stained in the same slide for comparison. Large degeneration forms characteristic of old cultures of globoid bodies may be seen. The clumping is due partly to the salt solution used in washing. Giemsa stain. $\times$ 1200.

FIG. 5. Washed globoid bodies showing phagocytosis in vitro. Giemsa stain. $\times$ 1200.

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